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Sample Preparation Methods

LARRY GREENFIELD AND THOMAS J. WHITE

Although there has been progress in simplifying the release and purification of bacterial or viral nucleic acids from clinical specimens, many research procedures are still unsuitable for the clinical laboratory and a universal automated method for use with any specimen has not yet been devised. In this chapter, we review some of the basic principles that have been learned to date which may guide and encourage the reader to develop further improvements that eliminate the requirements for hazardous solutions, centrifugations, and multiple steps. A variety of approaches which may be appropriate for certain specimens and pathogens but not for others are then described. Finally, each specimen type (e.g., whole blood, urine, sputum) is discussed with regard to specific protocols and pathogens.

Basic Principles

The ideal sample preparation method represents a trade-off between the requirements for the optimal method, the clinical specimen, and the target (Table 1). Although many of these considerations are interrelated, selection of a few crucial items helps define many others. Once the target organism is selected, the clinical pathogenesis of the infection generally dictates the appropriate specimen and number of microorganisms likely to be present. Determination of the desired assay sensitivity and the number of tests to be performed on the processed sample then dictates the required volume of specimen to be processed.

Sample Size Versus Target Copy Number

Microbiological culture as a "gold standard" has directed our selection of the appropriate specimen for many infectious diseases, e.g., blood or plasma for human immunodeficiency virus (HIV) and hepatitis C virus (HCV) and endocervical swabs for chlamydiae. For other pathogens, e.g., *Borrelia burgdorferi*, the optimal specimen for diagnosing each stage of infection has not yet been identified. For molecular diagnostic tests that are based on amplification, a single copy or molecule of the genetic target from the pathogen, if present in the reaction, can be detected in a fully optimized procedure (51, 70). To maximize the chance of diagnosing an infection, the largest convenient sample volume should be screened. However, since typical molecular diagnostic test reaction volumes are 100 μl or less, one is faced with a choice between complex target concentration steps (e.g., ethanol precipitation, nucleic acid target capture, and centrifugation) and lowered assay sensitivity. Hence, if no amplifiable target is detected

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Table 4. Fundamental goals of sample preparation protocols

Release of nucleic acid from bacteria, viruses, or fungi
Stabilization of nucleic acid against degradation
Removal of amplification inhibitors
Concentration of target into a small volume
Placement of target into an aqueous environment compatible with amplification

once an active infection has cleared. A study of the course of bacteremia by culture of specimens following oral trauma showed that viable bacteria were rapidly cleared from the blood (40). In one study of chlamydia infections following treatment, results of PCR and culture showed perfect concordance (23). However, these may be best-case scenarios; other studies have documented late persistence of nucleic acid (64). In some cases it is beneficial to detect dead microorganisms, e.g., when an inaccessible reservoir of live organisms sheds bacteria that are rapidly cleared. Many more longitudinal studies of treatment to cure for various diseases will be necessary to determine the clinical significance of DNAemia.

Overview of Approaches

Sample preparation methods can be divided into a number of generic steps (Table 4). The requirement for each step will depend on the organism and specimen. The release of nucleic acid may be easy for viruses and some bacteria (e.g., *Mycoplasma* species) but difficult for other bacteria (e.g., *Mycobacterium tuberculosis*) and fungi. RNA is more difficult to stabilize than DNA. More steps may be required to remove inhibitors from some specimens (e.g., sputum and blood) than from others (e.g., urine and CSF). Some specimens (e.g., sputum for *M. tuberculosis* and *Legionella pneumophila* and blood for sepsis) may require a greater degree of concentration than others (e.g., urethral swabs and urine for *Chlamydia* or *Gonococcus* species) to achieve the required sensitivity.

There are a variety of methods for the release of nucleic acid from microorganisms, including boiling in distilled water or PCR buffer (76), detergents with or without heat (76), sodium hydroxide with heat (13), freeze-thaw (15), SDS-proteinase K (51), perchloric acid (76), enzymes (30), sonication (15), and heat (55). Enzymatic digestion may be less desirable in that there may be components in the sample which prevent the action of the enzyme. For example, lysozyme has been used on liquified sputum (30), but sputum has a high content of mucopolysaccarides. Lysozyme is unstable following reduction (77) and forms complexes with dextrans and proteins (62). Many of the current and anticipated protocols require some method of separation for concentration of nucleic acid or removal of amplification inhibitors. Potential methods for separation include centrifugation, separation by magnetic particles, and separation by filtration.

Crude Lysis

The simplest sample preparation method would entail only a crude lysis. However, such a method would require a high concentration of target in the specimen and/or small amounts of amplification inhibitors. If no other separation step is included, the volume of the specimen to be processed is limited by the volume of the amplification reaction. Simple lysis methods typically use detergents such as SDS or Triton X-100, chaotropes such as guanidinium isothiocya-

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nate or sodium iodide, proteases such as proteinase K (which must be inactivated before the sample is added to the diagnostic reaction mixture) (42), substances such as saponin which lyse erythrocytes and leukocytes (e.g., the Wampole Isostat Microbial System), or heat (33). Such methods are generally suitable when the clinically significant number of infectious organisms per sample volume is large (e.g., *Chlamydia trachomatis* in endocervical swabs), so that the lysed specimen does not require significant further dilution (57). If the level of target is low, it is frequently necessary to remove amplification inhibitors by additional extraction (phenol-chloroform) steps or concentration of the target by alcohol precipitation. In addition, detergents are known to inhibit many enzymes, and high temperatures may result in degradation of nucleic acids (29).

Target Capture

Target capture or cycling offers the possible advantages of automation, universality for all specimens, and concentration of target into a small volume. This approach has been investigated by Gillespie et al. (36), Hunsaker et al. (44), and Lanciotti et al. (54). However, to date there are no published studies that demonstrate efficient capture and detection of fewer than 100 target molecules, and automated instruments and reagents for this approach are not yet commercially available. Derivatized magnetic particles can be coupled to oligonucleotide capture probes and combined with manual washing steps to remove extraneous materials (2, 19). These approaches have their own problems, though, since manual washing causes aerosols that may result in sample-to-sample contamination.

Other matrices have been tested for general adsorption of nucleic acids. Glass matrices, Sephadex, and diatomaceous earth bind nucleic acids in chaotropic solutions (11, 16, 59, 79). Following binding of the nucleic acids to the solid-phase matrix, the impurities and amplification inhibitors are removed by centrifugation and washing and the nucleic acids are eluted in an amplification-compatible buffer. Such approaches are promising since they are relatively simple, can be automated, and do not require hazardous reagents.

Finally, filtration may become a useful approach for certain kinds of specimens if it can be automated and made rapid (7a). Cost will be a problem unless disposable devices can be manufactured cheaply, and the requirement for a vacuum or centrifuge could be a burden for many laboratories.

Recommended Protocols for Various Specimens

Whole Blood

Even after it is decided that the desired specimen for a given target is blood, there still remain a number of choices: plasma, serum, whole blood, leukocyte fractions, etc. Furthermore, there is a choice of anticoagulants if the specimen is plasma: EDTA, heparin, or citrate. The anticoagulant used for plasma collection and the method of storage may affect the ability of the assay to detect the presence of target sequences (17, 80). Heparin was found to inhibit the activity of both murine leukemia virus reverse transcriptase and *Taq* DNA polymerase (46). In addition, the inhibitory effect of heparin does not appear to be removed by extraction of RNA by a modification of the acid-phenol-guanidinium method. For EDTA-containing tubes, it is recommended that the final concentration of EDTA be 1 to 2 mg/ml of blood (final concentration, 6.8 mM). For heparin-containing

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Sample Preparation Methods

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Although there has been progress in simplifying the release and purification of bacterial or viral nucleic acids from clinical specimens, many research procedures are still unsuitable for the clinical laboratory and a universal automated method for use with any specimen has not yet been devised. In this chapter, we review some of the basic principles that have been learned to date which may guide and encourage the reader to develop further improvements that eliminate the requirements for hazardous solutions, centrifugations, and multiple steps. A variety of approaches which may be appropriate for certain specimens and pathogens but not for others are then described. Finally, each specimen type (e.g., whole blood, urine, sputum) is discussed with regard to specific protocols and pathogens.

Basic Principles

The ideal sample preparation method represents a trade-off between the requirements for the optimal method, the clinical specimen, and the target (Table 1). Although many of these considerations are interrelated, selection of a few crucial items helps define many others. Once the target organism is selected, the clinical pathogenesis of the infection generally dictates the appropriate specimen and number of microorganisms likely to be present. Determination of the desired assay sensitivity and the number of tests to be performed on the processed sample then dictates the required volume of specimen to be processed.

Sample Size Versus Target Copy Number

Microbiological culture as a "gold standard" has directed our selection of the appropriate specimen for many infectious diseases, e.g., blood or plasma for human immunodeficiency virus (HIV) and hepatitis C virus (HCV) and endocervical swabs for chlamydiae. For other pathogens, e.g., *Borrelia burgdorferi*, the optimal specimen for diagnosing each stage of infection has not yet been identified. For molecular diagnostic tests that are based on amplification, a single copy or molecule of the genetic target from the pathogen, if present in the reaction, can be detected in a fully optimized procedure (51, 70). To maximize the chance of diagnosing an infection, the largest convenient sample volume should be screened. However, since typical molecular diagnostic test reaction volumes are 100 μl or less, one is faced with a choice between complex target concentration steps (e.g., ethanol precipitation, nucleic acid target capture, and centrifugation) and lowered assay sensitivity. Hence, if no amplifiable target is detected

Table 4. Fundamental goals of sample preparation protocols

Release of nucleic acid from bacteria, viruses, or fungi
Stabilization of nucleic acid against degradation
Removal of amplification inhibitors
Concentration of target into a small volume
Placement of target into an aqueous environment compatible with amplification

once an active infection has cleared. A study of the course of bacteremia by culture of specimens following oral trauma showed that viable bacteria were rapidly cleared from the blood (40). In one study of chlamydia infections following treatment, results of PCR and culture showed perfect concordance (23). However, these may be best-case scenarios; other studies have documented late persistence of nucleic acid (64). In some cases it is beneficial to detect dead microorganisms, e.g., when an inaccessible reservoir of live organisms sheds bacteria that are rapidly cleared. Many more longitudinal studies of treatment to cure for various diseases will be necessary to determine the clinical significance of DNAemia.

Overview of Approaches

Sample preparation methods can be divided into a number of generic steps (Table 4). The requirement for each step will depend on the organism and specimen. The release of nucleic acid may be easy for viruses and some bacteria (e.g., *Mycoplasma* species) but difficult for other bacteria (e.g., *Mycobacterium tuberculosis*) and fungi. RNA is more difficult to stabilize than DNA. More steps may be required to remove inhibitors from some specimens (e.g., sputum and blood) than from others (e.g., urine and CSF). Some specimens (e.g., sputum for *M. tuberculosis* and *Legionella pneumophila* and blood for sepsis) may require a greater degree of concentration than others (e.g., urethral swabs and urine for *Chlamydia* or *Gonococcus* species) to achieve the required sensitivity.

There are a variety of methods for the release of nucleic acid from microorganisms, including boiling in distilled water or PCR buffer (76), detergents with or without heat (76), sodium hydroxide with heat (13), freeze-thaw (15), SDS-proteinase K (51), perchloric acid (76), enzymes (30), sonication (15), and heat (55). Enzymatic digestion may be less desirable in that there may be components in the sample which prevent the action of the enzyme. For example, lysozyme has been used on liquified sputum (30), but sputum has a high content of mucopolysaccarides. Lysozyme is unstable following reduction (77) and forms complexes with dextrans and proteins (62). Many of the current and anticipated protocols require some method of separation for concentration of nucleic acid or removal of amplification inhibitors. Potential methods for separation include centrifugation, separation by magnetic particles, and separation by filtration.

Crude Lysis

The simplest sample preparation method would entail only a crude lysis. However, such a method would require a high concentration of target in the specimen and/or small amounts of amplification inhibitors. If no other separation step is included, the volume of the specimen to be processed is limited by the volume of the amplification reaction. Simple lysis methods typically use detergents such as SDS or Triton X-100, chaotropes such as guanidinium isothiocya-

nate or sodium iodide, proteases such as proteinase K (which must be inactivated before the sample is added to the diagnostic reaction mixture) (42), substances such as saponin which lyse erythrocytes and leukocytes (e.g., the Wampole Isostat Microbial System), or heat (33). Such methods are generally suitable when the clinically significant number of infectious organisms per sample volume is large (e.g., *Chlamydia trachomatis* in endocervical swabs), so that the lysed specimen does not require significant further dilution (57). If the level of target is low, it is frequently necessary to remove amplification inhibitors by additional extraction (phenol-chloroform) steps or concentration of the target by alcohol precipitation. In addition, detergents are known to inhibit many enzymes, and high temperatures may result in degradation of nucleic acids (29).

Target Capture

Target capture or cycling offers the possible advantages of automation, universality for all specimens, and concentration of target into a small volume. This approach has been investigated by Gillespie et al. (36), Hunsaker et al. (44), and Lanciotti et al. (54). However, to date there are no published studies that demonstrate efficient capture and detection of fewer than 100 target molecules, and automated instruments and reagents for this approach are not yet commercially available. Derivatized magnetic particles can be coupled to oligonucleotide capture probes and combined with manual washing steps to remove extraneous materials (2, 19). These approaches have their own problems, though, since manual washing causes aerosols that may result in sample-to-sample contamination.

Other matrices have been tested for general adsorption of nucleic acids. Glass matrices, Sephadex, and diatomaceous earth bind nucleic acids in chaotropic solutions (11, 16, 59, 79). Following binding of the nucleic acids to the solid-phase matrix, the impurities and amplification inhibitors are removed by centrifugation and washing and the nucleic acids are eluted in an amplification-compatible buffer. Such approaches are promising since they are relatively simple, can be automated, and do not require hazardous reagents.

Finally, filtration may become a useful approach for certain kinds of specimens if it can be automated and made rapid (7a). Cost will be a problem unless disposable devices can be manufactured cheaply, and the requirement for a vacuum or centrifuge could be a burden for many laboratories.

Recommended Protocols for Various Specimens

Whole Blood

Even after it is decided that the desired specimen for a given target is blood, there still remain a number of choices: plasma, serum, whole blood, leukocyte fractions, etc. Furthermore, there is a choice of anticoagulants if the specimen is plasma: EDTA, heparin, or citrate. The anticoagulant used for plasma collection and the method of storage may affect the ability of the assay to detect the presence of target sequences (17, 80). Heparin was found to inhibit the activity of both murine leukemia virus reverse transcriptase and *Taq* DNA polymerase (46). In addition, the inhibitory effect of heparin does not appear to be removed by extraction of RNA by a modification of the acid-phenol-guanidinium method. For EDTA-containing tubes, it is recommended that the final concentration of EDTA be 1 to 2 mg/ml of blood (final concentration, 6.8 mM). For heparin-containing

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